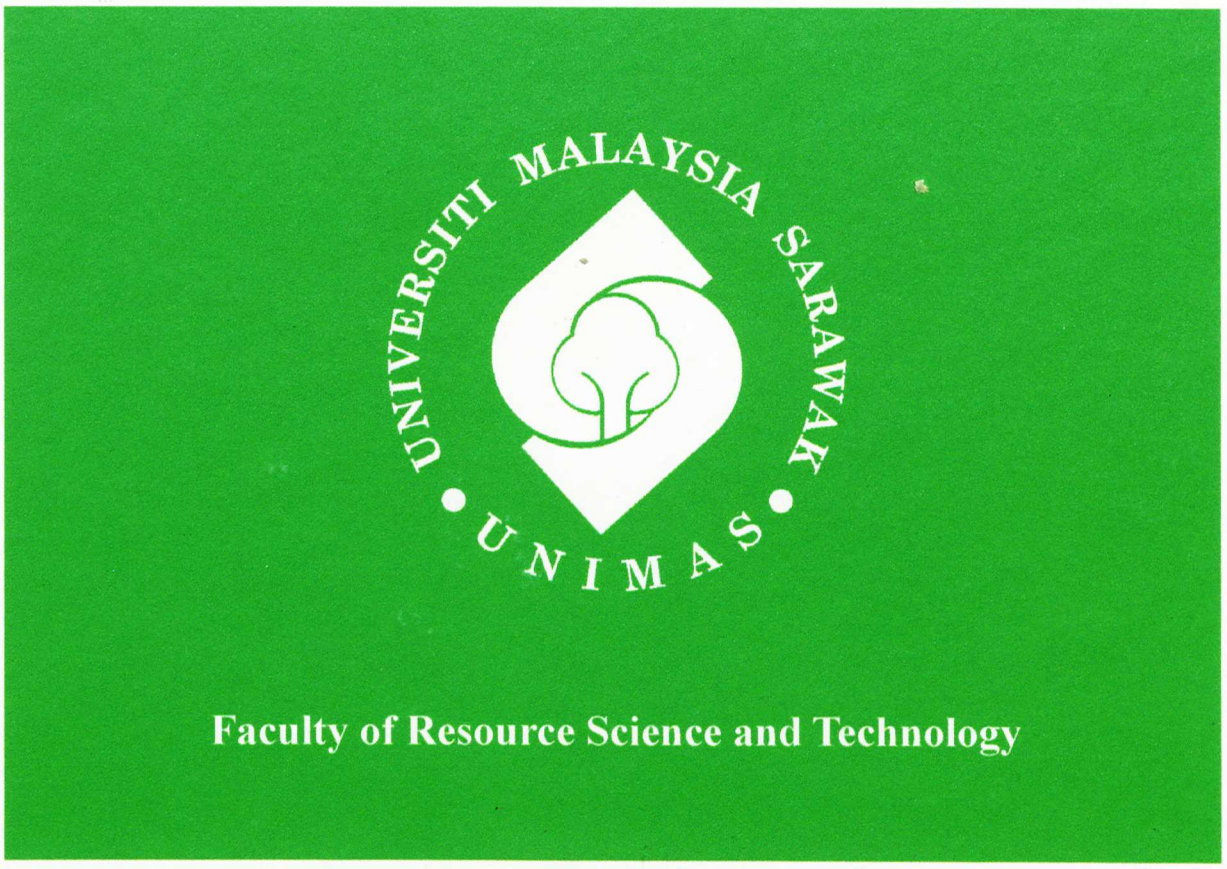

Heterologous Expression of Recombinant Cellulase in *Escherichia coli* Rosetta (DE3) and BL21 (DE3)

Casandra Kana Brooke (51377)

Bachelors of Science with Honours
(Resource Biotechnology)
2018

UNIVERSITI MALAYSIA SARAWAK

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HETEROLOGOUS EXPRESSION OF RECOMBINANT CELLULASE IN *ESCHERICHIA COLI* ROSETTA (DE3) AND BL21 (DE3)

Prepared by

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor Science with Honours
(Resource Biotechnology)

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HETEROLOGOUS EXPRESSION OF RECOMBINANT CELLULASE IN *ESCHERICHIA COLI* ROSETTA (DE3) AND BL21 (DE3)

CASANDRA KANA BROOKE

Resource Biotechnology

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ABSTRACT

The heterologous expression of cellulase gene from *Aspergillus sp.* in *E.coli* strains of Rosetta (DE3) and BL21 (DE3) has been studied to compare the recombinant protein production and to observe their activity, efficiency and production rate of recombinant cellulase. In previous study, an improved protein quality has been detected by using Rosetta, therefore further works need to be carried out to compare its protein production with BL21. The pFTAG-vector with the recombinant cellulase was used for expression studies and inserted into both Rosetta and BL21. Screening of cellullolyctic organism has failed to reveal the activity of recombinant cellulase as there is no growth detected on the media, as well as no formation of halo zone. Other than detection using the formation of halo zone, Bradford Assay and DNS assay was conducted to determine the cellulase activity of the bacterial in liquid medium. Recombinant cellulase from Rosetta was shown to have higher cellulase activity when the protein concentration was calculated using the standard graph. It was due to the fact that Rosetta has rare codons implemented inside them in order to yield higher protein production and better protein purity.

Key words: recombinant cellulase, Rosetta (DE3), BL21 (DE3), Bradford assay

ABSTRAK

Ekspresi heterologi gen selulase dari Aspergillus sp. dalam strain E.coli Rosetta (DE3) dan BL21 (DE3) telah dikaji untuk membandingkan pengeluaran protein rekombinan dan untuk melihat aktiviti, kecekapan dan kadar pengeluaran selulase rekombinan. Dalam kajian terdahulu, kualiti protein yang lebih baik telah dikesan dengan menggunakan Rosetta, oleh itu kerja lebih lanjut perlu dilakukan untuk membandingkan pengeluaran protein dengan BL21. Vektor pFTAG dengan selulase rekombinan diguinakan untuk kajian ekspresi dan dimasukkan ke dalam kedua-dua Rosetta dan BL21. Penyaringan organisma selulosa telah gagal untuk mendedahkan aktiviti selulase rekombinan kerana tidak ada pertumbuhan yang dikesan di media, serta tidak membentuk zon halo. Selain pengesanan menggunakan pembentukan zon halo, Bradford Assay dan pemeriksaan DNS dijalankan untuk menentukan aktiviti selulase bakteria dalam medium cair. Cellulase rekombinan dari Rosetta ditunjukkan mempunyai aktiviti selulase yang lebih tinggi apabila kepekatan protein dikira menggunakan graf standard. Ia disebabkan oleh fakta bahawa Rosetta mempunyai kodon yang jarang berlaku di dalamnya untuk menghasilkan pengeluaran protein yang lebih tinggi dan kesucian protein yang lebih baik

Kata kunci: selulase rekombinan, Rosetta (DE3), BL21 (DE3), Bradford assay

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LIST OF ABBREVIATIONS

AGE	Agarose gel electrophoresis
cm	Centimetre
CMC	Carbomethyl cellulose
EtBr	Ethidium bromide
kB	Kilobyte
LB	Luria Bertani
mL	Milliliter
mg	Miligram
mM	Millimetre
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
OD	Optical density
rpm	Rotation per minute
RE	Restriction enzyme
TAE	Tris-acetate-EDTA
µL	Microlitre
µg	Microgram

1.0 INTRODUCTION

Cellulase as an enzyme has become the focal biocatalyst due to its ability in breaking down cellulose molecules into simple sugars. Cellulose breakdown is considered to be economically important as it makes variety of plants readily available for consumption and its wide usage in chemical reaction. Their potential application in various industrial processes can be seen in cotton and paper manufacturing, starch industry, formulation of detergents, in juice extraction and as animal feed additives (Bhat, 2000). Phitsuwan et al. (2013) stated that cellulase enzymes are also being use in biotechnology and bioenergy fields, especially in the making of ethanol, butanol or other sugar fermentation products. The action of cellulase enzymes involves the cleavage of the 1,4-beta-D-glycosidic linkages in cellulose, hemicellulose, lichenin, and cereal beta-D-glucans and turn them into simpler monomers.

The Fungi genuses that are the native source of cellulases are Aspergillus, Trichoderma, Humicola, Penicillium and Phanerochaete (Singhania et al., 2010). Wood et al., (1992) and Lynd et al., (2002), in their study stated that cellulases have been manufactured inside bacteria as a substitute to reduce the cost for cellulase production. The manufactured of cellulase inside bacteria is called recombinant cellulase as it uses bacteria expression system to produce the protein. The advantages of recombinant protein are it contain highest purity by percentage, has specific activity, can be supply continuously and possess high level of consistency (Uppangala, 2010). Since recombinant cellulase is of important commercial value and often has high in demands in industry, the current method of expression will not be enough to produce the recombinant cellulase. Therefore, various researches aim to find ways to yield them efficiently in large quantities and in functional form.

In this study, two strains of *Escherichia coli,* BL21 (DE3) and Rosetta (DE3) has been chosen as host to express the production of recombinant cellulase. Among various expression hosts for production of heterologous protein, *Escherichia coli* remains the most attractive as it is properly-characterized, easy genetic manipulation, possessed many commercially available strains and vectors and high expression yields up to 50% of total recombinant protein (Chang et al., 2012). In the previous research, the effect of two *Escherichia coli* expression strains on the production of recombinant human protein fragments was evaluated (Tegel et al., 2009). It shows that production of recombinant protein in Rosetta (DE3) has better expression yield and possess significantly better purity of the protein product in comparison to BL21 (DE3). Thus, it is expected that Rosetta (DE3) will produce more recombinant cellulase when compare to BL21 (DE3). Both BL21 and Rosetta were used throughout this study but this time with different vector, pFTAG-cellulase which possess strong T7 promoter activity under IPTG induction. The ultimate aim of this study is to compare the recombinant protein produce in BL21 and Rosetta and to observe their activity, efficiency and production rate of recombinant cellulase.

The objectives of this research are:

1. To analyze the expression of recombinant cellulase in BL21 (DE3) and Rosetta (DE3)
2. To compare the expression of recombinant cellulase in BL21(DE3) and Rosetta (DE3)

2.0 LITERATURE REVIEW

2.1 Overview of Cellulase

Cellulase belongs to a group of enzymes that involves in the hydrolysis of β-1,4 linkage of cellulose chains. These cellulose molecules will be reduced to simple monomers such as beta glucose, short-chain polysaccharides and oligosaccharides. There are three main types of cellulose existence in nature such as endoglucanase, exoglucanase and β-glucosidase (Lynd et al., 2002). Main producer of enzyme cellulase are fungi, bacteria and protozoan. These organisms secrete cellulase in order to efficiently obtain energy from the breakdown of cellulose.

Kumar (2008), stated that the hydrolysis of cellulose involves the use of several types of cellulase enzymes in which they will perform different roles cooperatively. Some cellulase cut the cellulose chain into 4-5 glucoses at the middle site, some hydrolyze the chains into two unit glucoses, and some cleaves the chain into single glucose. According to SERVA Electrophoresis (n.d.), the cellulase enzyme works best in hydrolysis of cellulose at the optimum temperature of 40 – 50 °C and between pH 4 – 5 respectively.

2.1.1 Cellulase enzyme system

In cellulase enzyme system of microorganism, it uses two strategies to completely utilize their cellulase; discrete non-complexed cellulase and complex cellulase (Lynd et al., 2002). Generally, a set of individuals cellulases secretion contain carbohydrate-binding molecules (CBM) joined by a flexible linker peptide which may be placed at N-terminus or C-terminus of catalytic module. For anaerobic microorganism, it synthesizes cellulosomes that are bound to the cell surface of the microorganism (Bayer et al., 2004). But only a few of cellulosomes contain CBM, therefore most of them rely on the attachment of scaffoldin protein that contains CBM.

2.1.2 Application of cellulase

Cellulase enzyme has been applied widely in industry such as textile industry, bioethanol industry, food processing industry, animal feed industry, waste management and agricultural industry. In pulp and paper industry, cellulase enzyme is used for fiber properties biomodification to improve drainage and beatability in the paper mills before or after beating of pulp (Dienes, 2004). Successful application of cellulase can also be seen in biostoning of jeans and biopolishing of cottons in textile industry. In textile industry, cellulase-based treatment is used as the substitute of pumice stone and this has therefore had escalated the machine's productivity, reduce work intensity and environmental-friendly (Sukumaran et al., 2005 ; Singh et al., 2007). Another important contribution of cellulase can be seen in bioethanol industry as the technologies are readily available for the bioconversion of lignocellulosics to ethanol and other chemical products (Kuhad et al., 1997 ; Sun and Cheng, 2002). Several aspects have been geared to efficiently produce ethanol from lignocellulosic biomass; cellulase production optimization and the evolution of more productive cellulase-based catalysis system. Thus, due to its tremendous contributions in industrial process, cellulase enzyme has become the potential candidate for research.

2.2 Heterelogous cellulase production in bacterial expression system

The heterelogous expression of cellulase usually depends on the induction of relevant genes, thus it can be expressed efficiently when they are cultured on cellulosic subtrates under optimum condition with non-easily metabolized carbon source (Kotchoni, 2003). The cellulase expression can also be stimulated in rich nutrient media by using inducible or auto inducible promoters. These promoters play a key role in raising the expression of heterelogous cellulase.

This has been proven in strain of *Bacillus* for heterologous expression of alkaline cellulase under inducible sucrose promoter, sacB promoter in high nutrient media. The sacB promoter inserted inside *Bacillus* increased the expression level for heterologous cellulase production up to 20-fold as compared with expression under its own promoter (Liu, 2012).

Nevertheless, there are several issues on achieving cellulase expression in bacteria, especially *E.coli*. These issues are related with degradation of linker sequences in multi domain cellulases, inclusion bodies formation, wrong transportation across outer membrane and reduced specific activity of cellulase (Mittendorf, 1993). Hence, several strategies has been employed to counter these issues such as fusing a selected catalytic domain to CBM of another soluble cellulase, purification of inclusion bodies that contain cellulase, using specific substances such as urea and β-mercaptoethanol with subsequent refolding and changing the expression temperature (Koukiekolo et al., 2005).

2.3 *Escherichia coli* as expression host

Various organisms have been used as a host for secretion of protein such as bacteria, yeast, fungi, and unicellular algae. *Escherichia coli* are most known and widely used expression host as it consists of several advantages. First of all, it was chosen due to its rapid growth rate. If *E.coli* is grown under nutrient rich media and optimum condition, the doubling time will take around 20 minutes (Sezonov et al., 2007). This indicates that 1/100 dilution of starting culture has the ability to reach stationary phase in several hours. Despite this, the recombinant protein expression may cause metabolic burden on E.coli, thus reducing the generation time (Bentley et al., 1990). Not only that, it is easy to obtain high cell density cultures, thus strategies are designed using the physiology knowledge of this organism to enhance *E. coli* growth, even when

producing a recombinant protein (Choi et al., 2006). Next, media rich in nutrient sources can be prepare from readily available and inexpensive components. Another reason is the ease of transformability and genetic manipulation has made *E.coli* become the choice for propagation, manipulation and characterization of recombinant protein. Transformation of plasmid can be done in *E.coli* as fast as 5 minutes (Pope and Kent, 1996).

2.4 Construction of cellulase in pFTAG vector

The vector that will be use in this study pFTAG vector and is derived from pSTAG vector. The cellulase gene was designed from *Aspergillus sp.* and these gene are synthesised inside Rosetta and BL21.

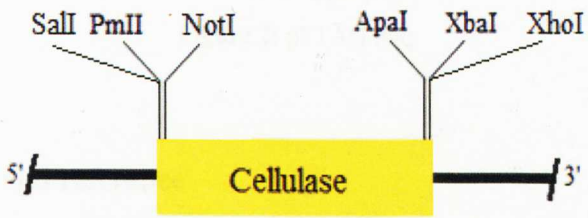

Figure 1: Expression cassette of Case

Cellulase (Case) was synthesized based from cellulase of *Aspergillus flavus*. A recombinant cDNA was synthesized to contain 555 amino acids. A *SalI*site (GTCGAC), *PmlI*site (CACGTG) and *NotI*site (GCGGCGGC) was introduced at 5' upstream Case gene sequence, while *ApaI* (GGG CCC), *XbalI* site (TCTAGA) and *Xho*l (CTCGAG) was introduced at 3' downstream of Case sequence as shown in **Figure 1**.

The Case was directionally-ligated into pFTAG vector via *SalI* and Xholsites. The pFTAG is derived from pSTAG vector with the inclusion of a FLAG signal. The pFTAG-Case was then cloned into *E.coli* BL21 and Rosetta strains.

2.4.1 pFTAG map and reference

This is a map of a pFTAG vector with size 2.8 kB. The vector consists of pUC ori sites, antibiotic-resistant to ampicillin site and f1 ori site. The promoter that is used to drive the expression of protein production is T7 promoter with the inclusion of FLAG signal.

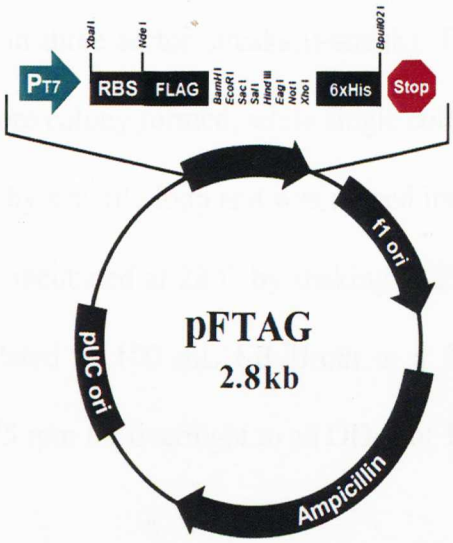

Figure 2: pFTAG map

2.4.2 pSTAG map and reference

This is a map of a pSTAG vector with size 2.9 kB. The vector consists of pUC ori sites, antibiotic-resistant to ampicillin site and f1 ori site. The promoter that is used to drive the expression of protein production is T7 promoter with the inclusion of S-Tag signal.

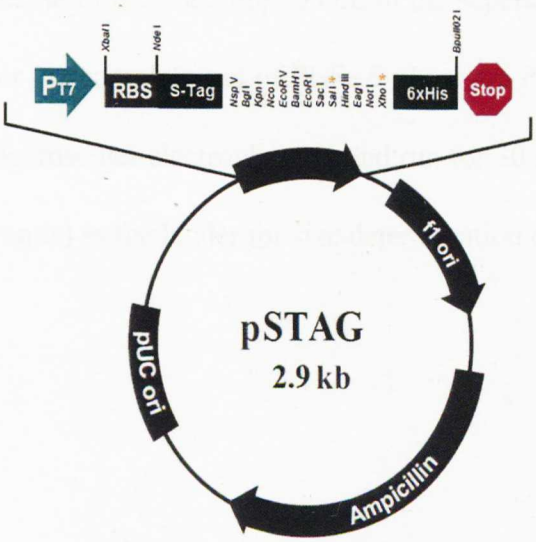

Figure 3: pSTAG map

3.0 MATERIALS AND METHODS

3.1 Culturing BL21 (DE3) and Rosetta (DE3)

BL21 and Rosetta strain were cultured by inoculating 1 colony from existing BL21 and Rosetta plate and streaked onto LB Agar in three sector streaks (t-streak). The plate was incubated in 30°C incubator for 3 days to let more colony formed, while single colony became the target. The single colony was observed, taken by a sterile loop and was dipped into 10 mL LB Broth in a 50 mL conical flask. The culture was incubated at 28°C by shaking at 250 rpm for 3 days. After 3 days, 1 ml of culture was inoculated in 100 mL LB Broth in a 250 mL conical flask and incubated at 28°C by shaking at 175 rpm for overnight to an OD_{600} of 1.3-1.5.

3.2 Plasmid extraction of pFTAG-cellulase from BL21 (DE3) and Rosetta (DE3)

Plasmid extraction was performed to check if BL21 and Rosetta contained the recombinant pFTAG-cellulase. First, the bacteria growing in LB Broth were vortexed for a few seconds in order to harvest them. About 250 µL of the bacteria was then pippetted and transferred to the microcentrifudge. The plasmid extraction was prepared according to GeneJet™ Plasmid Miniprep Kit. After finishing the plasmid extraction, 100 uL of the supernatant were then run for PCR at the thermos cycler. After the complete run of PCR, 5 µL of the PCR products contained in the tubes was loaded on the agarose gel-electrophoresis and run for 30 minutes at 100 V using 5 µL of 1 kB DNA Ladder (Vivantis) as the ladder for size determination of the loaded samples.

3.3 Screening of plasmid pFTAG-cellulase using colony PCR

Colony PCR was performed to observe if the transformants on LB Agar plates containing 100 µg/mL Ampicillin contain integrated DNA in it. One colony was picked from the plates and diluted with 10 µL of sterile water, and 1 µL was pipetted for PCR process. The list of primers and its sequences that were used is shown in the table below. The PCR Master Mix and parameters for PCR colony on pFTAG-cellulase is shown in **Table 1** (PCR Mix Volume for each tube).

Ingredients	Volume
GoTaq® Promega Master Mix	6.25 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Nuclease-Free Water	6.25 µl
Total	13.5 µl

Table 1: Master Mix for PCR colony for confirmation of inserts in pFTAG-cellulase

The parameters for PCR colony confirmation of inserts in pFTAG-cellulase is shown in **Table 2**.

Stage	Temperature (°C)	Time
Heat-lid	100	
Hot-Start	95	10 minutes
Heating (35X)	95	30 seconds
Annealing (35X)	58	30 seconds
Extension (35X)	72	3 minutes 15 seconds
Extending	72	7 minutes
Storage	4	5 minutes

Table 2: The PCR Parameters for pFTAG-cellulase

. After the complete run using thermos cycler for PCR colony analysis, 5 µL of the PCR products contained in the tubes was loaded on the agarose gel-electrophoresis and run for 30 minutes at 100 V using 5 µL of 1 kB DNA Ladder (Vivantis) as the ladder for size determination of the loaded samples.

3.4 Cell lysis of BL21 (DE3) and Rosetta (DE3)

3.4.1 Reagent preparation

The lysis buffer was prepared for 1 litre by dissolving 6 g of sodium phosphate, 372 mg of EDTA and 50 mL of glycerol in 900 mL deionized water. NaOH was added to adjust the pH to 7.4 and bring the volume to 1 litre. Before using the lysis buffer, 0.2 g of phenylmethylsulfonyl fluoride was added to the solution.

3.4.2 Cell lysis procedure

For every 1 ml sample, 100 uL of lysis buffer was added to the cell pellet and resuspended. Next, an equal volume of silica beads were added to the sample and vortexed. The sample was then placed on ice for 30 seconds. This step was then repeated for 8 – 10 times before the sample undergo centrifugation at 13,000 rpm for 10 minutes. The pellets were discarded and the supernatant was stored at 4 °C.

3.5 Bradford Protein Assay

3.5.1 Reagent preparation

Bradford reagent was prepared by dissolving 100 mg of Coomassie Blue G-250 in 50 mL of 95% ethanol. The solution was mixed with 100 mL of 85% phosphoric acid and added with distilled water until the solution becomes 1 litre. The reagent was filtered using Whatman no.1 filter paper to remove the precipitates just before use.

3.5.2 Standard Assay

Serial dilutions were prepared for both standards (Bovine Serum Albumin) and unknown samples and shown in **Table 3** and **Table 4.** Protein solutions are normally assayed in duplicate or triplicate. Bradford reagents of 1 mL were added to each tube, mixed well and were incubated at the room temperature for at least 5 minutes. Samples should not be incubated longer than 1 hour. The spectrophotometer was set to 595 nm and absorbance of the standards and samples were measured.

BSA dilution (µL)	ddH_2O (µL)	Total volume (µL)
20	80	100
40	60	100
80	20	100
100	0	100

Table 3: The serial dilutions of BSA

Sample dilution (µL)	Sample (µL)	ddH_2O (µL)	Total volume (µL)
100X	1	99	100
50X	2	98	100
25X	5	95	100

Table 4: The serial dilutions of sample

3.6 Dinitrosalicyclic Acid Assay

For preparation of DNS Assay, 20 g of dinitrosalicyclic acid, 4 g of phenol and 1 g of sodium sulfite were dissolved in 1 liter of 2% (w/v) NaOH solution and diluted to 2 litres with distilled water. Glucose was prepared by weighing 10 g of glucose powder and dissolved in 250 mL distilled water and used as a standard for this assay. Cellulose is use as a substrate and is added with the same volume of the samples. The standard and samples to be assayed were prepared in serial dilutions as shown in **Table 5** and **Table 6**. The standard and the samples were mixed with 1 ml of DNS reagent, added with cellulose and placed in 50 °C boiling water bath for 15 minutes and was cooled to room temperature. Then, both samples and standards were pipetted into cuvettes and the absorbance was read at 540 nm. The absorbance values were then changed to glucose equivalent using a standard graph by plotting glucose against the absorbance.

Glucose dilution (µL)	**ddH_2O (µL)**	Total volume (µL)
150	350	500
200	300	500
250	250	500
300	200	500
350	150	500

Table 5: The serial dilutions of glucose

Sample dilution (µL)	**Sample (µL)**	**ddH_2O (µL)**	Total volume (µL)
100X	5	495	500
50X	10	490	500
25X	125	475	500

Table 6: The serial dilutions of sample

3.7 Analyzing recombinant cellulase activity

3.7.1 M9 minimal plates agar preparation

The beaker was filled with 20 mL of distilled water. Then, 1.356 g of sodium monohydrogen phosphate, 0.6 g of mono potassium phosphate, 1.0 g of sodium chloride and 0.2 g of ammonium chloride were added into the beaker and diluted until 40 mL. In another beaker, 100 mL of distilled water was added along with 40 mL M9 salts (5X), 4 g CMC (2%) and 2 g of agar (1%). The solution from the first beaker was poured into the second beaker and was swirled to mix them. In a microcentrifuge tube, 2 tubes were prepared and 0.1203 magnesium sulphate was added in one tube and 0.1109 g of calcium chloride was added into another tube. Both tubes were filled with distilled water until it reached 1 mL. All the solutions were then autoclaved at 121 °C for 15 minutes. After autoclaving, the solutions were poured into petri dish plates and were left to solidify.

3.7.2 Screening of BL21 (DE3) and Rosetta (DE3) for cellulase activity

Individual transformed colonies were picked from LB Agar plates and transferred to minimal M9 plates containing 2% CMC. Cells were grown for 72 hours. The plates were then stained with 1% Congo red solution for 15 minutes and dye excess was removed with 1 M NaCl. Cellulase-producing clones were identified by the presence of a hydrolysis halo around the colony. The halo zone was observed to analyze the cellulase activity.